Volume 7 Number 8 1979 Nucleic Acids Research

Alkaline stability of guanosine and some of its derivatives modified by the carcinogen N-acetoxy-acetylaminofluorene

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Received 13 November 1979

ABSTRACT

The alkaline treatment of Guo, dGuo, dGMP and denatured DNA modified by N-acetoxyacetylaminofluorene (N-AcO-AAF) was performed in 0.1 M NaOH at 40°C. The kinetics of the reaction were followed by ultraviolet absorption and by chromatographic methods and were found different for the four products under study. Circular dichroism spectra show differences in the environment of acetylaminofluorene residue in these products. The alkaline treatment of Guo-AAF (and dGuo-AAF) leads to the formation of three products. These products were separated by thin layer chromatography and by HPLC and were characterized by spectroscopic methods. One is the already known unstable Guo-AF (and respectively dGuo-AF)(1). The other two products are relatively stable products of the transformation of Guo-AF (or dGuo-AF). These last ones present almost identical ultraviolet absorption spectra, but very different circular dichroism spectra.

INTRODUCTION

It is already established that when the carcinogens 2-acetylaminofluorene (2-AAF) and N-hydroxyacetylaminofluorene (N-OH-AAF) are injected to rats, they react with DNA. The majority of the product, about 80 %, is bound to DNA in the deacetylated form (DNA-AF) and only about 20 % in the acetylated form (DNA-AAF) (2,3). The adducts of DNA-AAF obtained *in vivo* and *in vitro* are well known : the N-(deoxyguanosin-8-yl)-AAF (1) and the 3-(deoxyguanosin-N^2-yl)-AAF (4).

In contrast, the DNA modified by the deacetylated form was less studied. However, recent studies show the importance of the *in vitro* mutagenicity of aminofluorene (5,6) and of the formation of aminofluorene as intermediate in the *in vitro* mutagenicity of AAF (7,8). The adducts of DNA-AF are not yet firmly identified. The instability of DNA-AF and of Guo-AF, already reported in the literature (9-12) makes it difficult to determine the nature of the adducts, which can be degraded *in vivo* or during the extraction or by enzymatic hydrolysis of DNA. The only aspect which is established is that AF gets bound *in vitro* in majority to guanine (13,14) but the precise sites of binding are not determined.

Our previous results also indicated the transformation in alkaline pH of AF residues bound to DNA. Moreover, we observed similar ultraviolet absorption spectra of alkali treated dDNA-AAF and dDNA-AF which suggest similar final reaction products (15). These observations led us to the study of the alkaline stability of AAF and AF modified monomers.

N-OH-AF reacts with native and denatured DNA at slightly acidic pH ; in the same experimental conditions no reaction was detected with the nucleosides or nucleotides (13,17) but the AF substituted monomers can be obtained by alkaline hydrolysis of the tertiary N-acetyl group of N-(guanosin-8-yl)-AAF (and respectively the AAF adducts of deoxyguanosine and deoxyguanosine-5'-monophosphate) (1,16).

In the present study we investigate the kinetics of the alkaline treatment of N-acetylaminofluorene adducts of guanosine, deoxyguanosine-5'-monophosphate and denatured DNA.

MATERIALS AND METHODS

Guanosine, deoxyguanosine and 5'-deoxyguanosine monophosphate are Sigma products. N-acetoxyacetylaminofluorene was synthesized starting from N-OH-AF (prepration previously described (15) with some minor modifications). N-AcO-(^{14}C)AAF was prepared from N-OH-AF and ^{14}C-acetic anhydride (CEA) according to the following procedure : a mixture of N-OH-AF (20 mg) and ^{14}C-acetic anhydride (1mCi) in 2 ml of dry ethyl acetate was stirred for 10 minutes. After evaporation of the solvent, the residue was washed with pretroleum ether, dissolved in 0.5 M NaOH (0.5 ml) and treated by 0.5 ml of acetic anhydride under nitrogen for 15 minutes. Extractions with ethyl ether (10 ml) of the reaction product gave upon evaporation *in vacuo* the labelled N-AcO-AAF. The latter was stored in ethanol under nitrogen at -20°C. GMP-AAF and dGMP-AAF (50 mg of nucleotide in 8 ml of buffer Na citrate 10^{-3} M, pH 7.5 were mixed with 50 mg of N-AcO-AAF in 8 ml ethanol) at 40°C for 3 hours, and purified on a DEAE cellulose column as previously described (16).

Guo-AAF and dGuo-AAF were prepared by dephosphorylation of the above products by alkaline phosphatase treatment at 37°C, pH 8.0 (Tris-HCl 10 mM) for 1 hour and subsequent HPLC purification. We employed a Jobin-Yvon HPLC apparatus Miniprep LC, with a Lichrosorb RP 18 (10 µ) column from Merck (L = 20 cm, Ø = 2 cm) ; the elution solvent was methanol-water 50/50 (v/v). The same device and elution system were used for the separation of the products resulting from alkaline treatment of Guo-AAF and dGuo-AAF. Radioactive Guo-AAF was prepared by direct reaction of (5'-^{3}H) guanosine (New England Nuclear ; specific activity 31 Ci/mmol) with N-AcO-AAF as previously described (18).

Denatured DNA-AAF and DNA-(^{14}C)AAF were prepared by direct reaction of DNA with N-AcO-AAF at 37°C for 3 hours at pH 7.0 (buffer 2×10^{-3} M Na citrate, 20 % ethanol). The DNAs were modified at 7 % of the nucleotides for DNA-AAF and at 13 % for DNA-(^{14}C)AAF.

The ultraviolet absorption measurements were carried out with a Cary 15 spectrophotometer. The circular dichroism measurements were performed with a Roussel-Jouan dichrograph 3. Thin layer chromatography was performed on Silicagel plates F 254 Merck with fluorescent marker. The chromatography system solvents were : System I : n-butanol-acetic acid-water 100:22:50 (v/v/v) for Guo-AAF and dGuo-AAF and System II : isopropanol-ammoniac 25 %-water 6:3:1 (v/v/v) for dGMP-AAF. The removal of acetyl group from the AAF residues linked to DNA was followed by the measurement of the radioactivity of dialyzed samples with a Beckman scintillation counter.

Guo-AAF, dGuo-AAF and dGMP-AAF were stored in a mixture of ethanol-2mM Na citrate, pH 7.0, 50:50 (v/v) under nitrogen at -20°C. The alkaline treatment was carried out in solutions of ethanol-2 mM Na citrate pH 7.0 5:100 (v/v) to which an 1 M NaOH solution was added under stirring.

RESULTS

1. Kinetics of alkaline treatment

Alkaline treatment of Guo-AAF, dGuo-AAF, dGMP-AAF and denatured DNA-AAF (dDNA-AAF) was done at 40°C in 0.1 M NaOH. The evolution of ultraviolet absorbance of Guo-AAF and of dDNA-AAF (in the region where DNA does not absorb) are presented in figure 1. Similar evolutions were observed for dGuo-AAF and dGMP-AAF (results not shown). The variation with time of the ratio between the absorbance at 320 nm and that at one isosbestic wavelength (303 nm for the monomers-AAF and 306 nm for dDNA-AAF) is presented in figure 2. The results of thin layer chromatograms for the monomers and radioactivity measurements for dDNA-(^{14}C)AAF (radioactivity carried by the acetyl group -see Materials and Methods) performed at given time intervals during the reaction are presented in parallel on figure 2.

In all cases one observes first a time interval in which absorbance at e.g. λ = 320 nm increases and at λ = 285 nm decreases ($A_{320}/A_{\lambda isosbestic}$ is increasing). This phase corresponds to the disappearance of the acetylated form in the thin-layer chromatograms and to a decrease of radioactivity in the dialyzed DNA-AAF samples. Disappearance of the acetylated form occurs slower in dDNA-AAF than in dGMP-AAF, slower in dGMP-AAF than in dGuo-AAF and slower in dGuo-AAF than in Guo-AAF. During this phase one observes for all monomers the appearance of a product (product I) with Rf superior to Rf of the acetylated

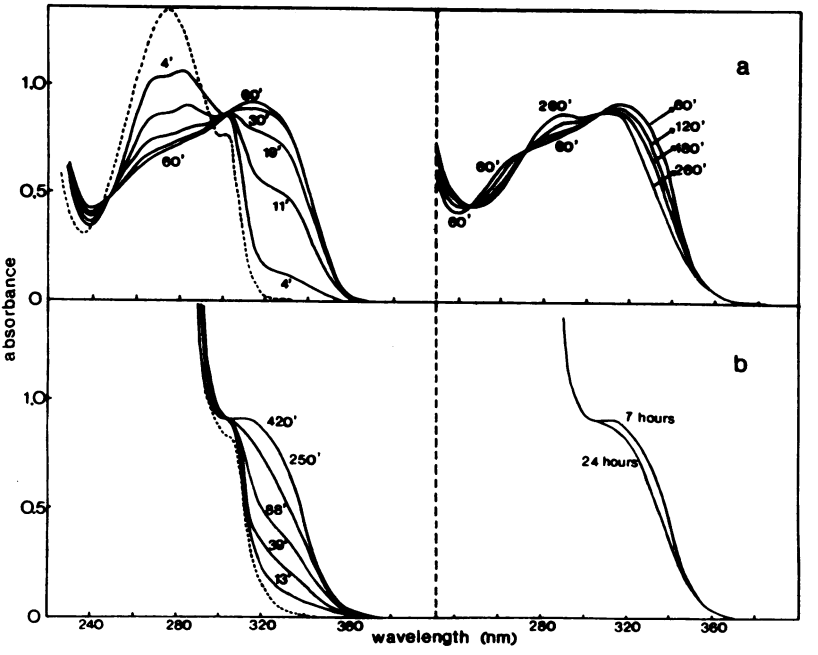

Figure 1 - Evolution of ultraviolet absorption spectra during alkaline treatment in 0.1 M NaOH, 40°C of a) Guo-AAF, b) dDNA-AAF (7 %). Concentrations of about 3.5×10^{-4} M (AAF) residues. The numbers indicated on the spectra represent the time of reaction in minutes. ---- Spectra of products at neutral pH, 20°C.

form. For dGuo-AAF a second product appears (product II) and immediately afterwards a third one (product III). For Guo-AAF, a second product is also appearing and a third one is slightly visible. One obtains during this phase isosbestic point values of 303 and 248 nm for Guo-AAF, dGuo-AAF, dGMP-AAF and 306 nm for dDNA-AAF.

This step is followed by an other one in which A_{320} is decreasing and A_{285} is increasing ($A_{320}/A_{\lambda isosbestic}$ is slightly decreasing) in the case of the modified monomers. By visual observation of the chromatograms one observes during this phase an increase of intensity of the spots corresponding to products II and III in disfavor of product I. The values of isosbestic points observed in the first phase are very slightly shifted (1-2 nm) in this last phase. The R_f values of all three products of the alkaline treatment of Guo-AAF, dGuo-AAF and dGMP-AAF are given in Table I.

A more quantitative analysis of the reaction was done using (5'-^{3}H) Guo-

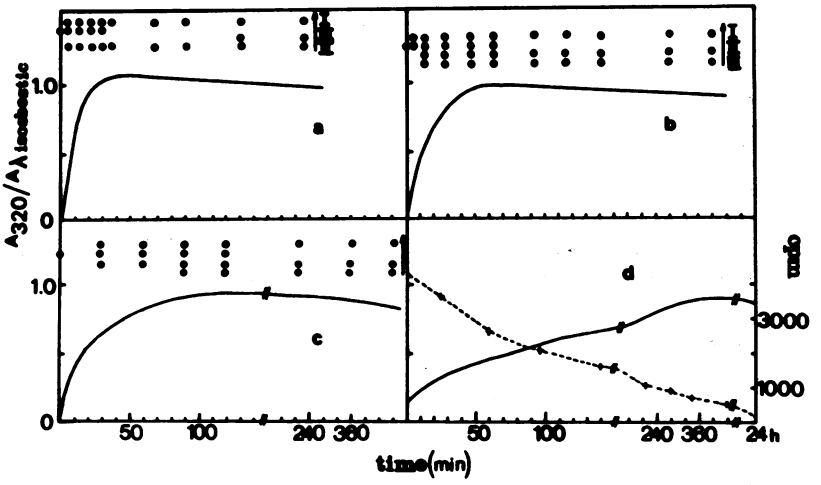

Figure 2 - Variation of $A_{320}/A_{\lambda isosbestic}$ with time calculated from the U.V. absorption spectra for a) Guo-AAF, b) dGuo-AAF, c) dGMP-AAF, d) DNA-AAF. Same conditions as in Fig. 1. In a), b) and c) the tops of the figures are schematic representations of thin-layer chromatograms of samples taken at given time intervals. In d) the dashed line represents the variation of radioactivity of dialized DNA-AAF samples as a function of time. The numbering I, II and III of the products is done in order of appearance of spots in chromatograms.

AAF. The radioactivity of the spots obtained by thin layer chromatography was determined and the results are presented in Fig. 3. The appearance of product I and its transformation in products II and III are clearly observed in this experiment. The proportions of products I, II and III after 12 hours of alkaline treatment are of about 86 % product II, 12 % product III and 2 % product I.

The first appearing product (I) in the case of Guo-AAF has an U.V. absorp-

Table I - Values of R_f in the thin-layer chromatograms of the reaction products (chromatography system solvents described in Materials and Methods).

	Guo-AAF (syst.I)	dGuo-AAF (syst.I)	dGMP-AAF (syst.II)
Initial product	0.61	0.67	0.72
Product I	0.68	0.71	0.76
Product II	0.44	0.56	0.69
Product III	0.52	0.47	0.67

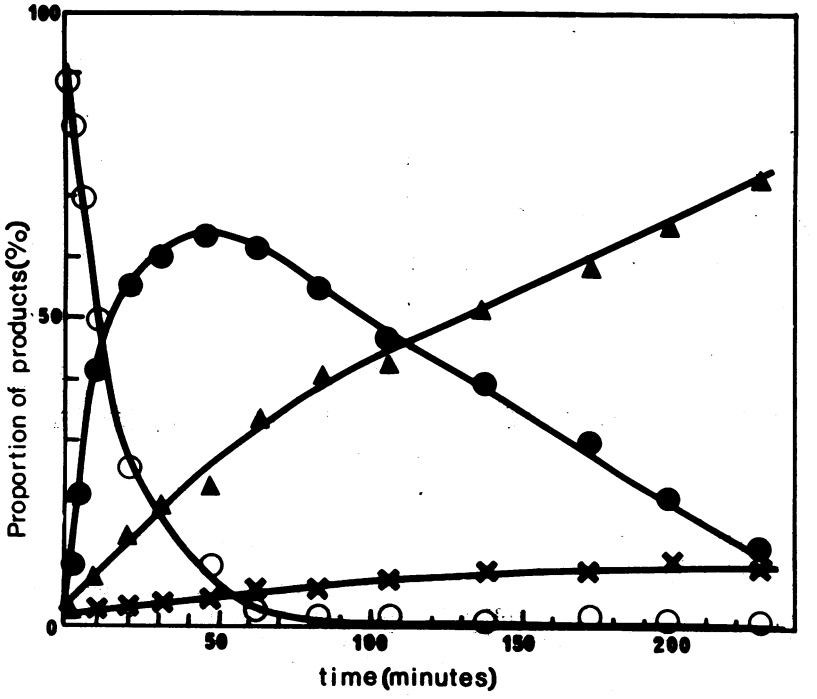

Figure 3 - Relative proportions of Guo-AAF and the products of its alkaline treatment as a function of time. Same conditions as in Fig. 1. —o— initial product, —●— product I -Δ- product II, -x- product III.

tion spectrum identical to the one already published for Guo-AF (1). This product was isolated from the reaction mixture on a LH 20 column (elution with a linear gradient of 0.02 M ammonium carbonate-ethanol) and its stability was studied. In 0.1 M NaOH at 40°C the same type of evolution was observed as in the second part of the alkaline treatment of Guo-AAF (Figure 1a, right). By chromatography this evolution corresponded to the appearance of the spots characteristic of products II and III. Products II and III were stable when isolated and incubated further on for 1 hour in alkaline conditions.

In the case of alkaline treatment of dDNA-AAF the second step of the kinetics in which A_{320} decreased was also observed (Figure 1b). Moreover, we recall here an earlier result which shows that dDNA-AF (obtained by direct reaction of N-OH-AF with DNA in acidic pH) seems to behave in the same manner -decrease of A_{320}- when incubated in alkaline conditions (15).

2. Circular dichroism spectra of initial products

The circular dichroism spectra of Guo-AAF, dGuo-AAF, dGMP-AAF and dDNA-AAF at neutral pH and in 0.1 M NaOH at 4°C (in order to avoid deacetylation), as well as the spectra of unmodified monomers and dDNA, in the same conditions are presented in figure 4.

3. Ultraviolet absorption and circular dichroism spectra of the three reaction products in the case of Guo-AAF and dGuo-AAF

Separation of the three reaction products was performed by thin-layer chromatography (in system I) or by HPLC for both Guo-AAF and dGuo-AAF. The three products obtained in each case were characterized by ultraviolet absorption and circular dichroism spectra. The results are presented in figure 5. The ε values were calculated from the absorption spectra considering the value of ε(Guo-AAF) and ε(dGuo-AAF) at 302 nm, pH 7.0 and 20°C at 22,000 M^{-1} cm^{-1} (16). The presence of an isosbestic point (302 nm) over the first period of the reaction which shifts only very slightly over the second phase of reaction,

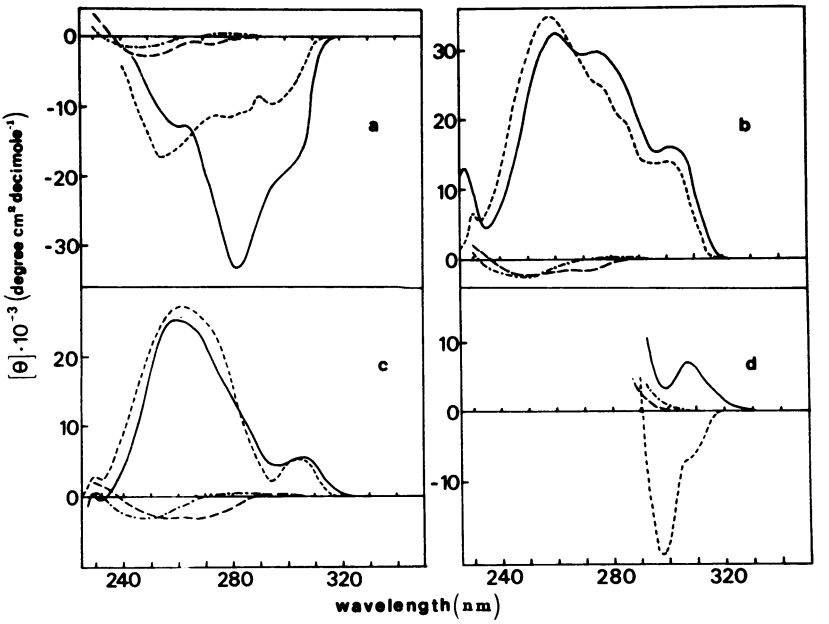

Figure 4 - Circular dichroism spectra of a) Guo-AAF, b) dGuo-AAF, c) dGMP-AAF, d) dDNA-AAF at 4°C in ---- neutral pH, ——— 0.1 M NaOH. The corresponding unmodified monomers Guo, dGuo, dGMP and dDNA in —·—·— neutral pH and — — — 0.1 M NaOH.

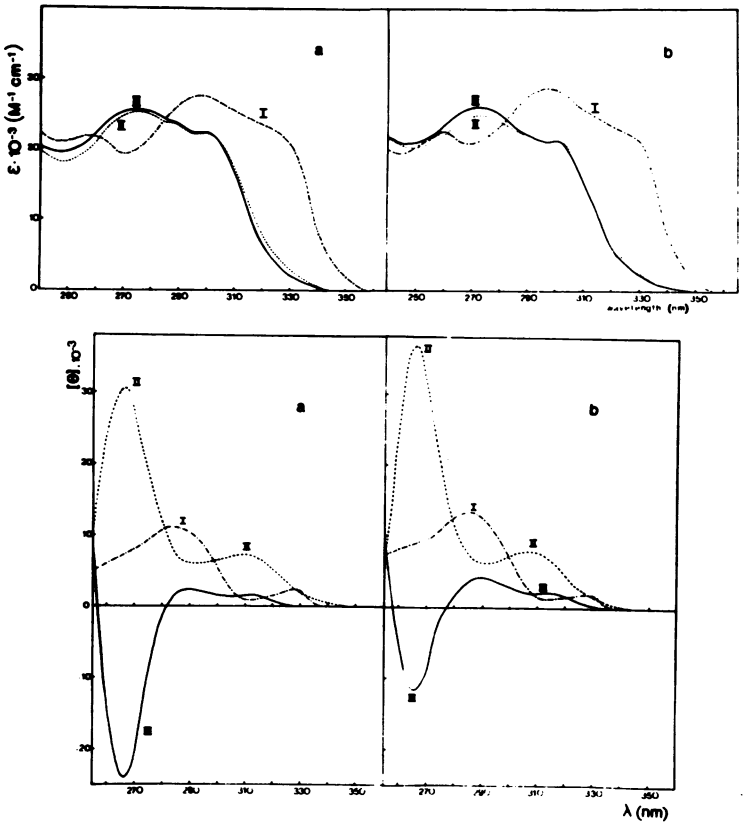

Figure 5 - Molar extinction coefficients and molar ellipticity as a function of wavelength for the three products obtained by alkaline treatment of a) Guo-AAF, b) dGuo-AAF in 2 mM Na citrate, pH 7.0 at 20°C.

suggested to us that the three products of the reaction have all almost the same ε at 302 nm in 0.1 M NaOH and 40°C.

We did the approximation that all these products have the same extinction coefficient at this wavelength. Thus from the spectra of each product at neutral pH and 20°C and in alkaline pH and 40°C we calculated the ε values which are presented in figure 5.

DISCUSSION

The results presented here are showing differences and similarities in the alkaline treatment of Guo-AAF, dGuo-AAF, dGMP-AAF and dDNA-AAF.

The differences are concerning the rate of deacetylation of the products.

Thus dDNA-AAF is deacetylated slower than dGMP-AAF, this last one slower than dGuo-AAF and this very last one slower than Guo-AAF. There are at least two factors which can influence the kinetics of the reaction : the conformation and the electrical charge of the products. From circular dichroism spectra one can deduce conformational aspects. The CD spectra of Guo-AAF and dGuo-AAF are very different which proves a drastic difference in their conformations. On the other hand, the CD spectra of dGuo-AAF and dGMP-AAF are similar in shape (intensities are however different) which suggests similar conformation of the two products. Therefore one can assume that the different rates of deacetylation are due to different stabilities of acetyl group resulting from differences in the conformations and in the electrical charge of the products. The case of dDNA-AAF is much more complex since more factors are involved, e.g. conformation of the nucleoside residues,influence of the neighboring bases and polyelectrolyte effects.

The similarities concern the number and the spectral characteristics of products obtained by the alkaline treatment. In each of the three cases (Guo-AAF, dGuo-AAF and dGMP-AAF) we obtained three reaction products.

The first one (product I) can be identified to Guo-AF (or dGuo-AF or dGMP-AF) from the ultraviolet absorption spectra similar to the one already published (1). At alkaline pH this product is instable and gets transformed in two other products (II and III). In spite of the almost identical ultraviolet absorption spectra of products II and III they can be easily differentiated by their circular dichroism spectra. Because of the similar ultraviolet absorption and circular dichroism spectra of the two products I (deoxy and ribo), of the two products II (deoxy and ribo) and of the two products III (deoxy and ribo) we can conclude that the same transformation occurs at alkaline pH for both Guo-AAF and dGuo-AAF. It is striking that for all the AF-reaction products, the circular dichroism spectra are no more sensitive to the nature of the sugar. It mainly reflects the interaction between the base and the fluorene residue. This might suggest that in the case of AAF-conjugates, the acetyl group-sugar interactions play an important role in the conformation of the products.

In the case of dDNA-AF obtained by direct reaction of N-OH-AF and dDNA the spectrum on the fluorene region corresponds well to the one of product I.

When incubated in alkaline conditions it shows an evolution similar to the one observed for the transformation of product I in products II and III (see Figure 9 of (15)). The evolution of dDNA-AAF in alkaline conditions tends toward the same limit. Due to all these similarities we suggest that the alka-

line treatment of Guo-AAF, dGuo-AAF, dGMP-AAF, dDNA-AAF and dDNA-AF will conduct at the limit to a mixture of the two AF-modified products II and III. We notice that this was already shown for dGuo-AAF by a different technique (19). A chemical structure for these two products implying opening of the imidazole ring of guanosine was very recently proposed (20).

In conclusion, it is tempting to speculate that such a transformation of dGuo-AF can occur *in vivo* due to a local alkaline pH. It is obvious that this will perturb seriously the properties of DNA containing these residues. This is an attractive explanation of the mutagenicity of aminofluorene residues.

Acknowledgements

This work was partially supported by the Délégation Générale à la Recherche Scientifique et Technique, Contract n° 77.7.1349.

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